

珍稀濒危植物金花茶炭疽病新病原菌的鉴定

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摘要: 为明确金花茶炭疽病原菌的种类, 进而为金花茶炭疽病的有效防治提供理论基础, 对金花茶炭疽病样本进行了病原菌分离和纯化, 获得疑似病原菌菌株。选取其中2株代表菌株 JH01 和 JH04 开展致病性测定, 发现其均能引起金花茶炭疽病发生。通过形态学观察, 并采用最大似然法 (Maximum Likelihood, ML) 和贝叶斯法 (Bayesian Inference, BI) 构建多基因片段系统进化树, 最终将菌株 JH01 和 JH04 鉴定为果生炭疽菌 *Colletotrichum fructicola*, 是引起金花茶炭疽病的新病原菌。

关键词: 金花茶; 炭疽病; 果生炭疽菌; 病原菌

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Identification of the pathogen of anthracnose on the endangered and rare plant *Camellia petelotii*

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Abstract: In order to clarify the taxonomy of anthracnose pathogens on *Camellia petelotii* (Merr.) Sealy and provide theoretical basis for the effective control of the disease, the pathogens in the samples of *C. petelotii* anthracnose were isolated and purified, and the suspected pathogenic fungus were obtained. Two representative strains, JH01 and JH04, were selected for pathogenicity testing and both were able to induce the symptoms of anthracnose. Through morphological observation, as well as the construction of multi-gene fragment phylogenetic trees using Maximum Likelihood (ML) and Bayesian Inference (BI) methods, the strains were identified as *Colletotrichum fructicola*, which caused anthracnose on *C. petelotii*.

Key words: *Camellia petelotii* (Merr.) Searly; anthracnose; *Colletotrichum fructicola*; pathogen

金花茶 *Camellia petelotii* (Merr.) Sealy 是隶属于山茶科 Theaceae Mirb. 山茶属 *Camellia* L. 的国家二级保护珍稀濒危植物, 主要分布在我国广东、广西、湖南、贵州等地。金花茶的花色金黄, 具蜡质光泽, 有“植物界大熊猫”“茶族皇后”“东方魔茶”“国宝神茶”等美称(黄燮才, 1994; 何桂玲等, 2016; 黄昌艳等, 2016)。金花茶除了观赏价值外, 还具有较高的药用价值和生态价值。金花茶化学成分丰富, 含有黄酮、皂甙、多糖等多种生理活性成分和钾(K)、硒(Se)、钼(Mo)、锌(Zn)等多种矿物元素(秦小明等, 2005; 彭靖茹和甘志勇, 2009); 金花茶的提取物具有抗肿瘤、抗炎、抗氧化、调血脂、降血糖、抗肥胖、降血压、抗过敏、抗衰老等药理作用(曾英港等, 2022); 金花

茶对 SO₂、氟化物等污染气体也具有较强的吸收净化能力(赵鸿杰等, 2021)。

目前, 金花茶已报道的叶部病害有6种, 包括炭疽病 *Colletotrichum camelliae* Masee、赤叶枯病 *Phyllosticta* sp.、藻斑病 *Cephaleuros virescens* Kunze、煤烟病 *Meliola camelliae* (Catt.) Sacc.、茶红锈藻病 *Cephaleuros parasicas* Karst 和日灼病, 其中炭疽病发生最为普遍和严重(曹季丹和梁盛业, 1986; 周建良和彭珍宝, 1995; 吴儒华等, 2008; 谢玲等, 2009; 梁惠凌等, 2012; 何桂玲等, 2016; 程照明等, 2018)。

近年来, 金花茶炭疽病在广东省广州市石门国家森林公园种植的金花茶苗木上频发, 已造成一定的危害。为明确引起该炭疽病的病原菌种类, 本研

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究对病害症状进行观察,对病原菌进行分离、纯化,验证其致病性后,结合形态学特征和多基因系统发育学分析鉴定了病原菌种类,为制定有效的金花茶炭疽病防控措施提供理论依据。

1 材料与方 法

1.1 试验材料

金花茶患病叶片于2023年9月采集自广东省广州市石门国家森林公园(113°48'50" E, 23°37'18" N)。

1.2 病原菌分离与纯化

采用组织分离法(方中达,1998)对采集的病叶进行病原菌的分离。将叶片的病健交界处病组织切成5 mm × 5 mm 小块,用75%乙醇浸泡30 s后,再用3%的次氯酸钠消毒1 min,无菌水漂洗3次后,于无菌滤纸上晾干,随后将组织块置于PDA培养基上28℃培养,待菌落长出后,挑取菌落组织制片镜检,并用灭菌牙签挑取菌落边缘菌丝接种于新的PDA培养基上,获得纯化的病原菌。纯化后的真菌于4℃下斜面保存备用。

1.3 致病性测定

基于菌落形态将1.2中分离得到的菌株JH01和JH04作为代表性菌株测定其致病性。选取大小大致相同的金花茶离体叶片,用无菌水清洗干净,待叶片表面水分晾干后置于含有湿润滤纸的培养皿中,用灭菌的接种针在叶片主脉右侧中部穿刺1次,将直

径6 mm的病原菌菌饼正面接种于穿刺孔上,以接种相同大小的无菌PDA作为对照,接种后将培养皿置于28℃培养箱中培养,每个处理重复3次。观察记录发病情况,待接种叶片发病后从发病部位再次分离纯化病原菌,鉴定对比是否与接种菌株一致,若一致,则表明接种的菌株即为致病菌。

1.4 病原菌形态学鉴定

将接种至PDA平板上纯化的菌株,置于28℃培养箱中恒温培养7 d,观察并记录菌落颜色、气生菌丝等形态特征;待其产孢后,挑取孢子制作玻片在显微镜下观察,随机选取100个分生孢子进行形态、大小及有无隔膜的观察和记录。

1.5 病原菌系统发育分析

采用真菌基因组DNA提取试剂盒(Solarbio, Cat#D232000)提取病原菌DNA,对菌株JH01和JH04核糖体DNA内转录间隔区(ITS)、甘油醛-3-磷酸脱氢酶(GAPDH)、肌动蛋白(ACT)、几丁质合成酶1(CHS1)和β微管蛋白(TUB2)基因片段进行PCR扩增,扩增引物详见表1。PCR反应体系(25 μL):正向与反向引物(10 μmol)各2 μL、2×Taq PCR MasterMix(含染料)12.5 μL、DNA模板2 μL、ddH₂O 6.5 μL。菌株扩增条件参照于琳等(2022)的研究。反应结束后,取5 μL PCR产物在1%琼脂糖凝胶进行电泳检测。条带单一且清晰的PCR产物送至北京擎科生物科技有限公司广州分公司进行测序。

表1 扩增基因所需引物序列

Tab. 1 PCR primers for genes amplification

基因 Gene	引物名称 Primer	引物序列 (5'-3') Sequences	参考文献 Reference
ITS	ITS1	TCCGTAGGTGAACCTGCGC	White et al., 1990
	ITS4	TCCTCCGCTTATGATATGC	
GAPDH	GDF1	GCCGTCAACGACCCCTTCATT	Guerber et al., 2003
	GDR1	GGGTGGAGTCGACTTGAGCATGT	
ACT	ACT-512F	ATGTGCAAGCCGGTTTCGC	Carbone and Kohn, 1999
	ACT-783R	TACGAGTCCTTCTGGCCCAT	
CHS1	CHS-79F	TGGGCAAGGATGCTTGAAGAAG	O' Donnell and Cigelnik, 1997
	CHS-354R	TGGAAGAACCATCTGTGAGAGTTG	
TUB2	T1	AACATGCGTGAGATTGTAAGT	Aveskamp et al., 2009
	TUB4Rd	CCRGAYTGRCCRAARACRAAGTTGTC	

序列拼接在SeqMan(DNASTAR Lasergene Software Suite for Sequence Analysis)中进行,拼接好的序列在NCBI数据库上进行blastn分析,并下载本属真菌的序列,与测定的菌株相关基因序列进行系统发育分析,所用序列信息详见表2。在PhyloSuite1.2.2中采用最大似然法(Maximum Likelihood, ML)和贝叶斯法(Bayesian Inference, BI)构建多基因片段联合系统发育进化树(Guindon et al., 2010; Ronquist et al., 2012; Nguyen et al., 2015; Zhang et al., 2020)。

2 结果与分析

2.1 金花茶炭疽病症状及病原分离

金花茶炭疽病发病时初现红色小点,后向外周围扩展成片状,病斑边缘具明显的黑红色线纹,病健交接处呈暗紫红色(图1)。发病末期炭疽病斑转为灰白色,其上密布病原菌黑色子实体(图1b, c),严重时导致整片叶片干枯脱落。通过组织分离法分离共获得15个菌株,其中炭疽菌共4株,分离率达到26.67%。

表2 炭疽菌属系统发育分析使用的参考菌株及其 GenBank 登录号

Tab. 2 GeneBank accession numbers of strains for phylogenetic analysis of *Colletotrichum*

菌种 Species	菌株号 Culture	基因登录号 GeneBank accession number				
		<i>ITS</i>	<i>GAPDH</i>	<i>ACT</i>	<i>CHS-1</i>	<i>TUB2</i>
<i>Colletotrichum aenigma</i>	ICMP 18 608	JX010244	JX010044	JX009443	JX009774	JX010389
<i>Colletotrichum alatae</i>	CBS 304.67*=ICMP 17 919	JX010190	JX009990	JX009471	JX009837	JX010383
<i>Colletotrichum camelliae</i>	GT6	LC738932	LC738940	LC738944	LC738948	LC738936
<i>Colletotrichum clidemiae</i>	ICMP 18 658	JX010265	JX009989	JX009537	JX009877	JX010438
<i>Colletotrichum conoides</i>	CAUG17	KP890168	KP890162	KP890144	KP890156	KP890174
	ICMP 12 568	JX010166	JX009946	JX009529	JX009762	JX010388
	ICMP 17 787	JX010164	JX009958	JX009439	JX009807	JX010401
	ICMP 17 788	JX010177	JX009949	JX009458	JX009808	JX010408
	ICMP 18 613	JX010167	JX009998	JX009491	JX009772	JX010394
	BL15	MZ373180	MZ405674	MZ405668	MZ405671	MZ405677
<i>Colletotrichum fruticola</i>	CBS 125 397*=ICMP 18 646	JX010173	JX010032	JX009581	JX009874	JX010409
	BL16	MZ373181	MZ405675	MZ405669	MZ405672	MZ405678
	LZ3	MZ373182	MZ405676	MZ405670	MZ405673	MZ405679
	JH01	PP789730	PP854716	PP864634	PP864636	PP864638
	JH04	PP789731	PP854717	PP864635	PP864637	PP864639
<i>Colletotrichum gloeosporioides</i>	IMI 356 878*=ICMP 17 821=CBS 112 999	JX010152	JX010056	JX009531	JX009818	JX010445
<i>Colletotrichum hebeiense</i>	K3	KF156863	KF377495	KF377532	KF289008	KF288975
<i>Colletotrichum higginsianum</i>	IMI 349 061=CPC 19 379	KM105184	KM105535	KM105394	KM105254	KM105464
<i>Colletotrichum horii</i>	C1180.1	GQ329690	GQ329681	JX009438	JX009752	JX010450
<i>Colletotrichum jiangxiense</i>	LC3463=CGMCC 3.173 63=LF687	KJ955201	KJ954902	KJ954471	-	KJ955348
<i>Colletotrichum kahawae</i>	IMI 319 418*=ICMP 17 816	JX010231	JX010012	JX009452	JX009813	JX010444
<i>Colletotrichum musae</i>	CBS 116 870*=ICMP 19 119	JX010146	JX010050	JX009433	JX009896	HQ596280
<i>Colletotrichum nupharicola</i>	CBS 470.96*=ICMP 18 187	JX010187	JX009972	JX009437	JX009835	JX010398
<i>Colletotrichum proteae</i>	CBS 132 882	KC297079	KC297009	KC296940	KC296986	KC297101
<i>Colletotrichum psidii</i>	CBS 145.29*=ICMP 19 120	JX010219	JX009967	JX009515	JX009901	JX010443
<i>Colletotrichum queenslandicum</i>	ICMP 1 778*	JX010276	JX009934	JX009447	JX009899	JX010414
<i>Colletotrichum salsolae</i>	ICMP 19 051*	JX010242	JX009916	JX009562	JX009863	JX010403
<i>Colletotrichum siamense</i>	CBS 125 378*=ICMP 18 642	JX010278	JX010019	GQ856775	GQ856730	JX010410
<i>Colletotrichum syzygicola</i>	DNCL021	KF242094	KF242156	KF157801	-	KF254880
<i>Colletotrichum theobromicola</i>	CBS 124 945*=ICMP 18 649	JX010294	JX010006	JX009444	JX009869	JX010447
<i>Colletotrichum tibetense</i>	ICMP 4 832*	JX010269	JX009952	JX009520	JX009898	JX010442
<i>Colletotrichum tropicale</i>	CBS 124 949*=ICMP 18 653	JX010264	JX010007	JX009489	JX009870	JX010407

注: *表示该菌株为模式菌株; “-”表示该菌株没有相应的基因登录号。

Note: * indicates that this strain is a type strain; indicates that the strain has no corresponding genebank accession number.

2.2 致病性测定

基于菌落形态选取代表性菌株 JH01 和 JH04 接种至健康叶片, 3 d 后叶片开始发病, 5 d 叶片上形成明显不规则形状的病斑。病斑呈棕褐色或黑褐色, 病斑部位软化并凹陷, 接种无菌 PDA 的叶片对照均未发病。对发病叶片进行再分离, 所得病原菌与原接种病原菌形态特征一致, 符合科赫氏法则, 说明 JH01 和 JH04 均为金花茶炭疽病的病原菌。2 株菌株中, JH01 病斑呈轮纹状、JH04 病斑呈不规则形状(图 2)。

2.3 病原菌形态

菌落在 PDA 上正白色, 呈疏松绒毡状, 菌丝略厚且蓬松, 背面淡黄色(图 3a, c), 菌株 JH04 比菌株 JH01 菌丝生长速度略快且更为茂盛。孢子堆呈橘红色。显微镜下分生孢子单孢, 透明, 细长胶囊形, 两端钝圆(图 3b, d)。菌株 JH01 分生孢子大小为 $(8.0 \sim 17.8) \mu\text{m} \times (2.8 \sim 6.1) \mu\text{m}$, 平均 $12.8 \mu\text{m} \times 4.2 \mu\text{m}$ ($n=100$)。菌株 JH04 分生孢子大小为 $(9.1 \sim 20.6) \mu\text{m} \times$

$(3.4 \sim 7.4) \mu\text{m}$, 平均 $15.6 \mu\text{m} \times 5.1 \mu\text{m}$ ($n=100$), 菌株 JH04 分生孢子略大于菌株 JH01。根据形态特征将其鉴定为炭疽菌属 *Colletotrichum* Cda。

2.4 系统发育分析

菌株 JH01 和 JH04 基因组 DNA 的 *ITS*、*GAPDH*、*ACT*、*TUB2* 和 *CHS1* 基因片段经扩增和测序, 得到的大小分别为 530、272、285、700 和 282 bp。基于 *ITS*、*GAPDH*、*ACT*、*TUB2* 和 *CHS-1* 多基因联合(总长为 2 069 bp)构建的 ML 系统发育树和 BI 系统发育树中(图 4), ML 树与 BI 树得到的拓扑结构一致, 拓扑结构以 BI 树为例, 菌株 JH01 和 JH04 与果生炭疽菌聚为独立的一支并获得强烈支持($PP=100$, $MLBP=94$), 确定其为果生炭疽菌。

3 讨论

金花茶炭疽病于 1995 年由周建良和彭珍宝首次



图1 金花茶炭疽病症状

Fig. 1 Symptoms of anthracnose on *C. petelotii*

注: a. 金花茶炭疽病症状; b-d. 病原菌在叶尖上产生的黑色子实体。

Notes: a. Symptoms of anthracnose; b-d. Type acervuli was observed on a leaf tip lesion.



图2 不同菌株接种金花茶离体叶片5 d 病斑形态

Fig. 2 Symptoms of detached leaves of *C. petelotii* inoculated with strain JH01 and JH04 for 5 d

在湖南省南岳树木园发现危害并报道, 谢玲等 2009 年在广西防城金花茶国家级自然保护区发现有金花茶炭疽病危害, 且两地发现的金花茶炭疽病的病原菌均为油茶炭疽菌 *Colletotrichum camelliae* Masee (周建良和彭珍宝, 1995; 谢玲等, 2009)。本研究通过分离, 结合形态学及分子生物学分析, 将引起广州市金花茶炭疽病的病原菌鉴定为果生炭疽菌, 以前鲜有该病原菌引起金花茶炭疽病的报道。研究结果对后续开展金花茶炭疽病的有效防治提供参考依据。

果生炭疽菌和油茶炭疽菌引起金花茶炭疽病症状相同 (周建良和彭珍宝, 1995; 谢玲等, 2009; 程照明等, 2018), 初发病时均呈现红色小点, 后向周围扩展

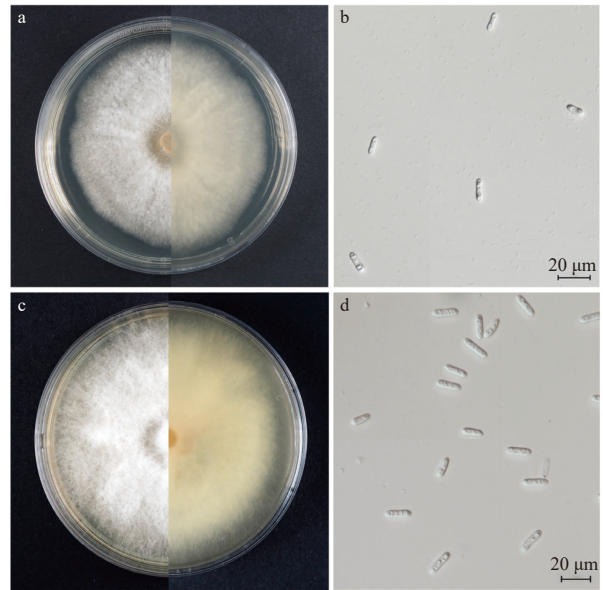


图3 菌株 JH01 和 JH04 的菌落和孢子形态特征

Fig. 3 Morphological characteristics of colonies and spores of strain JH01 and JH04

注: a, c 分别为菌株 JH01 和 JH04 在 PDA 上生长 5 d 的菌落形态; b, d 分别为菌株 JH01 和 JH04 的分生孢子。

Notes: a, c are colony morphology of strain JH01 and JH04 grown on PDA for 5 days, respectively; b, d are conidial morphology of strain JH01 and JH04, respectively.

成片状, 后期病斑会逐渐变为灰白色, 上布黑色子实体。本研究中观察到的炭疽病主要从叶尖开始变褐色, 逐步向叶片中部浸染, 灰褐色或灰白色的病斑逐步扩大。仅从金花茶炭疽病发病症状并不能区分果生炭疽菌和油茶炭疽菌导致的炭疽病。

果生炭疽菌属于胶孢炭疽菌复合种 *Colletotrichum gloeosporioides* Complex (Weir et al., 2012), 2009 年首次在泰国北部小粒咖啡 *Coffea arabica* L. 上被发现 (Prihastuti et al., 2009), 但其作为内生菌被报道较少 (Rojas et al., 2010; Liu et al., 2015; Xue et al., 2019), 更多的是作为病原菌被报道, 目前已报道危害约 90 种寄主植物, 涉及蔬菜辣椒 *Capsicum* spp.、青菜 *Brassica rapa* var. *chinensis* (L.) Kitam., 水果果实莲雾 *Syzygium samarangense* (Blume) Merr. & L. M. Perry、杧果 *Mangifera indica* L.、甜柿 *Diospyros kaki* Thunb., 果树叶片柑橘 *Citrus reticulata* Blanco、李 *Prunus salicina* Lindl. 及茶 *Camellia sinensis* (L.) O. Ktze.、油茶 *Camellia oleifera* Abel、海南坡垒 *Hopea hainanensis* Merr. & Chun 和橡胶树 *Hevea brasiliensis* (Willd. ex A. Juss.) Müll. Arg. 等经济林木 (李河等, 2017; 李杨秀等, 2018; 林春花等, 2018; 覃丽萍等, 2020; 谭祥丰等, 2020; 亓政良等, 2023; 任立超等, 2023; Peng et al., 2012; Diao et al., 2017; Mongkolporn and Taylor, 2018; Xue et al., 2019; Yu et al., 2022)。

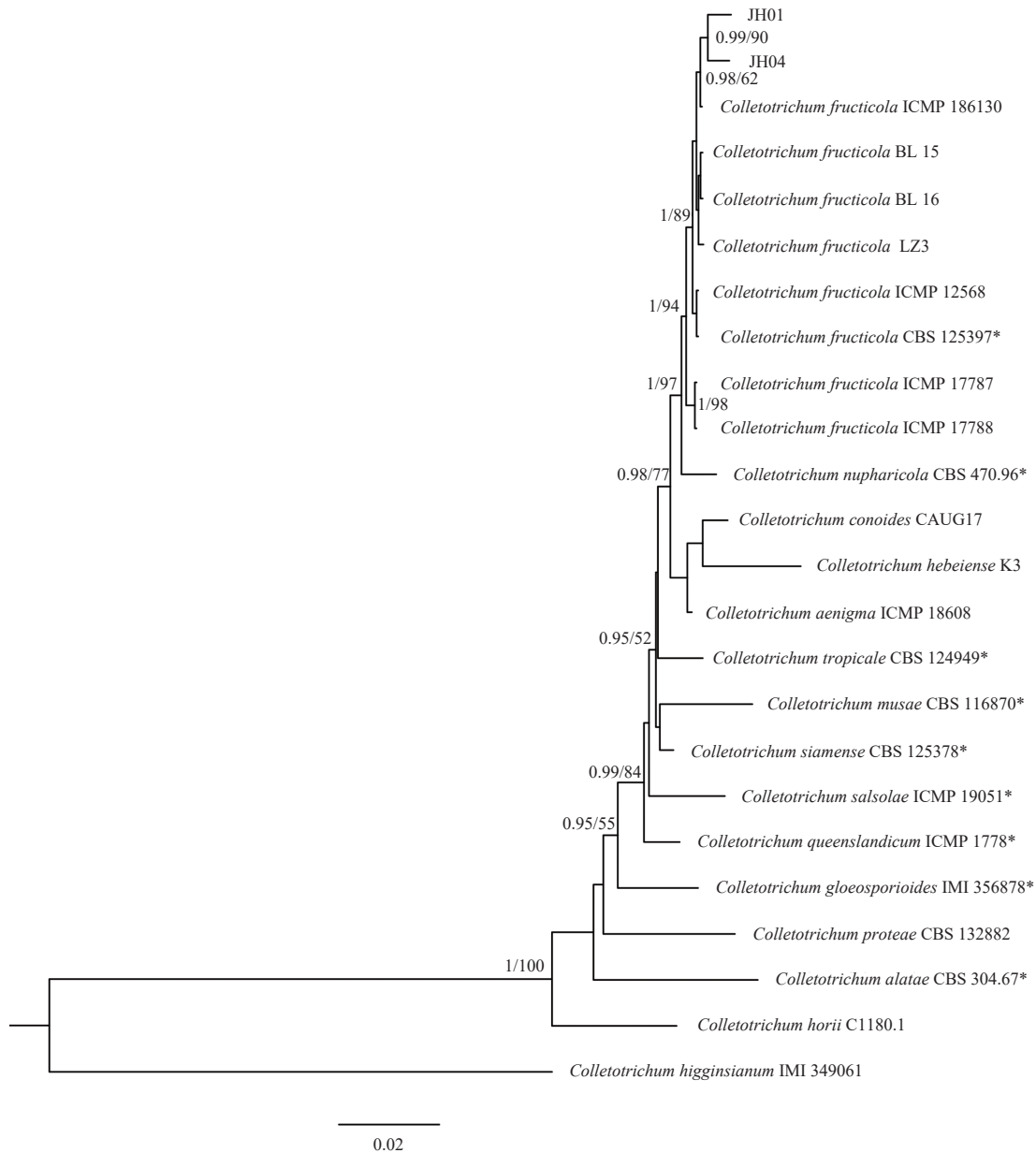


图4 基于 *ITS*、*GAPDH*、*ACT*、*TUB2* 和 *CHS-1* 多基因构建的 BI 树

Fig. 4 Phylogenetic tree constructed based on multiple genes including *ITS*, *GAPDH*, *ACT*, *TUB2*, and *CHS-1* using the Bayesian inference method

注: *表明该菌株为模式菌株。图中仅显示 BI 后验概率 ≥ 0.90 的节点, 节点上的值从左到右依次是: BI 后验概率/ML 法自举值。

Notes: * indicates that this strain is a type strain. Only nodes with Bayesian inference posterior probability greater than or equal to 0.90 are shown in the figure, The values on the nodes from left to right are: Bayesian inference posterior probability/Maximum Likelihood bootstrap value.

在 Prihastuti 等 (2009) 的研究中, 果生炭疽菌在 PDA 上生长的菌落最初为白色, 后期随着菌丝的生长, 菌落中心变为灰色至灰绿色, 孢子大小为 $(9.7 \sim 14.0) \mu\text{m} \times (3.0 \sim 4.3) \mu\text{m}$ ($n=180$)。本研究中金花茶炭疽病原菌后期菌落始终呈现白色绒毡状, 分生孢子略大, 这可能是地域差异、寄主植物不同等原因导致的。

随着分子生物学的发展, ITS 序列越来越多地应用于物种的识别和鉴定, Schoch 等 (2012) 认为其能很好地识别真菌种内和种间的变异。Weir 等 (2012) 研究中表明 ITS 序列能有效识别复合种, 但不能很好

识别出复合种内的差异, 复合种内 ITS 区域的碱基变化数量较少, 物种往往仅有 1~2 个碱基的差异。所以本研究采用多基因联合片段构建系统发育树, 能较好地对接孢炭疽菌复合种内的物种进行区分和有效识别。

4 结论

本研究通过致病性测定结合形态学鉴定和分子生物学鉴定, 从广东省广州市石门国家森林公园种植的金花茶炭疽病病叶样本中分离得到一种金花茶

炭疽病的新病原菌——果生炭疽菌。有关该病原菌的遗传和表型特征、致病性以及与其他几种炭疽病在侵染金花茶致病过程中的相关性等有待进一步研究, 以期为该病害在生产上的精准高效防控奠定基础。

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